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In 1996, the U.S. Congress mandated the development of a screening program for endocrine-disrupting chemicals (EDCs) using validated test systems. Subsequently, the Endocrine Disruptor Screening and Testing Advisory Committee recommended the development of a standardized amphibian assay for tier 2 testing of EDCs. For that reason, a tier 2 testing protocol using Xenopus (Silurana) tropicalis and a 30-week, flow-through exposure to the antiandrogen flutamide from state 46 tadpoles through sexually mature adult frogs were developed and evaluated in this pilot study. The endpoints for this study included measurements of frog body lengths and weights, liver weights, ovary/egg mass weights, testicular and ovarian histopathology, plasma vitellogenin levels, and notes on any abnormalities observed at necropsy. Increasing exposure concentrations to flutamide caused significant increases in frogs with no recognizable gonadal tissue and increased body and liver weights in male frogs, whereas the body lengths and weights decreased significantly in female frogs. Important issues must be resolved before a tier 2 amphibian assay can be further developed and validated, including the establishment of baseline values in the controls for the parameters under study; the

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Hazard/Risk Assessment

A NOVEL AMPHIBIAN TIER 2 TESTING PROTOCOL: A 30-WEEK EXPOSURE OF *XENOPUS TROPICALIS* TO THE ANTIANDROGEN FLUTAMIDE

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Abstract—In 1996, the U.S. Congress mandated the development of a screening program for endocrine-disrupting chemicals (EDCs) using validated test systems. Subsequently, the Endocrine Disruptor Screening and Testing Advisory Committee recommended the development of a standardized amphibian assay for tier 2 testing of EDCs. For that reason, a tier 2 testing protocol using *Xenopus* (*Silurana*) *tropicalis* and a 30-week, flow-through exposure to the antiandrogen flutamide from stage 46 tadpoles through sexually mature adult frogs were developed and evaluated in this pilot study. The endpoints for this study included measurements of frog body lengths and weights, liver weights, ovary/egg mass weights, testicular and ovarian histopathology, plasma vitellogenin levels, and notes on any abnormalities observed at necropsy. Increasing exposure concentrations to flutamide caused significant increases in frogs with no recognizable gonadal tissue and increased body and liver weights in male frogs, whereas the body lengths and weights decreased significantly in female frogs. Important issues must be resolved before a tier 2 amphibian assay can be further developed and validated, including the establishment of baseline values in the controls for the parameters under study; the maintenance, measurement, and timing of exposure concentrations; and the development of additional biomolecular markers of effect. This study demonstrated the feasibility of conducting long-term EDC exposure studies using *X. tropicalis*.

Keywords—Endocrine disruption Amphibian Flutamide *Xenopus tropicalis*

INTRODUCTION

The U.S. Environmental Protection Agency has been mandated to develop a screening program for endocrine-disrupting chemicals (EDCs) using validated test systems and has assigned a high priority to the development and standardization of tier 2 testing. Since a standardized amphibian test is considered important and a sufficiently comprehensive and established method has not been identified, the Amphibian Development and Reproduction Assay, which confirms and characterizes the effects of a potential EDC at various concentrations, has been recommended.

A substantial body of literature exists describing metamorphosis, growth, and reproduction in frogs. Much of our knowledge regarding chromosome replication [1], chromatin and nuclear assembly, control of the cell cycle [2], in vitro reconstruction of cytoskeletal dynamics, signaling pathways, mechanisms of early fate decisions [3], patterning of the basic vertebrate body plan, and early organogenesis [4] was acquired from years of research using the African clawed frog, *Xenopus laevis*, in both basic research and short-term, endocrine toxicity studies [5–8]. In comparison, *X. laevis* has been shown have responses to endocrine disruptors similar to *Rana pipiens* [9] when exposed to atrazine and *Rana catesbeia* when exposed to 4-*tert*-octylphenol [10]. Although *X. laevis* has been a widely used model in cellular and molecular biology, many believe the western clawed frog, *X. tropicalis*, has a greater potential as an experimental model for a number of reasons [11–13]. The basic developmental biology of *X. tropicalis* is very similar to *X. laevis*, and *X. tropicalis* responds similarly when exposed toxicants in the Frog Embryo Teratogenesis Assay-Xenopus model compared to *X. laevis* [14], but *X. tropicalis* reaches sexual maturity within four to six months, while *X. laevis* reaches sexual maturity in two to three years [13]. In addition, the diploid genome of *X. tropicalis* was chosen for sequencing rather than the pseudotetraploid genome of *X. laevis* [15].

Working together, several U.S. government agencies developed protocols to optimize the breeding and rearing of the amphibian *X. tropicalis* under aquatic flow-through conditions that are suitable for long-term laboratory exposure to EDCs. Protocols for the necropsy and histopathology of *X. tropicalis* following chronic exposure to an EDC were also developed. To test these protocols, we conducted a pilot study that exposed *X. tropicalis* for 30 weeks to five concentrations of the antiandrogen flutamide in a flow-through system, starting immediately after hatch and continuing through sexual maturity. In the current report, we describe the results of the pilot study and provide recommendations for further protocol development and standardization.

MATERIALS AND METHODS

Animals and husbandry

Laboratory well water used for rearing and testing all U.S. Army Center for Environmental Health Research (USACEHR)

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aquatic organisms was formulated and processed on-site. Well water pumped from a 550-foot-deep well passed through a carbon filtration system before being mixed at a 1:1 ratio with tap water that had also been carbon filtered and then treated by reverse osmosis. The mixed water was aerated in a holding tank, filtered through a 10-μm particle filter, heated to 25 ± 2°C, and distributed to all aquatic test systems.

The embryos for the study were obtained in-house from *X. tropicalis* (Nigerian strain) frogs bred at the USACEHR for three generations prior to the study. The original source of the frogs was the University of Virginia (Charlottesville, VA, USA). Adult frogs were housed in 18.9-L aquaria (20 frogs/tank) prior to mating and were acclimated to their environment for several months. The frogs were fed both Advanced Tadpole Diet (ATD) and Nasco® pellets (Fort Atkinson, WI, USA) twice a day. Water temperature was maintained at 25 ± 2°C [16], and a 12:12-h light:dark cycle was maintained.

Ten pairs of adult frogs were induced to mate by injecting 200 IU (males) and 250 IU (females) of Chorulon, a human chorionic gonadotropin preparation (Provet, Kansas City, MO, USA), into the dorsal lymph sac using a 1-ml syringe with a 26-gauge needle. Mating pairs were housed overnight in the dark using 4-L polycarbonate chambers containing laboratory well water at 25 ± 2°C. The following morning, a clutch of embryos that appeared to have the highest viability and least amount of necrosis and unfertilized eggs was selected from one mating pair. The embryos were degelled in 2% L-cysteine (pH adjusted to 8) and rinsed three times in laboratory well water before being placed in 4-L polycarbonate breeding chambers (~800 embryos per chamber) containing 1 L of processed laboratory well water. The embryos were maintained in total darkness in the incubator at a water temperature of 25 ± 2°C. After 24 h of incubation, the chambers were examined, dead embryos were removed, and an additional liter of laboratory well water was added to each chamber. At or before Nieuwkoop and Faber (NF) stage 46 [17] (48 h posthatch), the early tadpoles were viewed under a dissecting microscope to assess their development. Any abnormal tadpoles were culled, and normal tadpoles were randomly transferred, in groups of five, to glass aquaria until each test tank contained 40 tadpoles, for a total of 400 tadpoles. The 18.9-L glass aquaria contained 15 L of laboratory well water. The outside bottoms of the aquaria were painted white to enhance visual observations.

Once transferred into the test tanks, the tadpoles were fed 0.4 g of a Sera® Micron (Sera, Hiensberg, Germany)/Nasco (SMN) powder mixture (50:50, w/w) suspended in 10 ml of Milli-Q® (Millipore, Billerica, MA, USA) treated water four times a day (three times a day on weekends). At posthatch day 17, the SMN powder mixture was increased to 0.6 g four times a day until day 27, when the tadpoles reached NF stage 57 and had usable hind legs. At this point, the tadpoles received 0.5 g of ATD two times a day along with 0.6 g of the SMN powder mixture four times a day. The SMN powder was gradually replaced with ATD until ≥99% of the tank organisms had reached NF stage 66, signifying the end of metamorphosis. At this stage, which was day 41, the ATD completely replaced the SMN powder mixture, and the frogs then received ATD four times a day and Nasco pellets four times a day. During the 30-week study, frogs were fed sufficient food to minimize food searching behavior after feeding while leaving a minimum of uneaten food in the tanks.

Water was distributed to the test aquaria at a rate of 300 ml per 5-min interval (5.4 tank volumes/day) from a flow through diluter apparatus. Aquaria were held in a stainless-steel water bath held at a temperature of 25.5 ± 1.2°C. Water temperature was checked daily in a control tank and was recorded continuously in the same control tank throughout the study with a strip chart and temperature probe. Dissolved oxygen, pH, and conductivity were measured weekly in each of the tanks and had means (ranges) of 3.22 (0.01–6.7) mg/L, 7.2 (6.3–7.8), and 0.63 (0.17–1.2) ms/cm², respectively. Alkalinity, hardness, and un-ionized ammonia were measured weekly in a control and a high dose tank, and their means (ranges) were 204 (38–206) mg/L as $CaCO_3$, 190 (24–304) mg/L as $CaCO_3$, and 0.039 (0.003–0.171) mg/L, respectively. During testing, a 12:12-h light:dark cycle was used with an average light intensity of 322 lx at the test tank water surface. Light intensity was measured weekly until transparent lids were placed on the tanks at week 8; after that, no further light measurements were taken. Tanks were siphoned three times per week to control the amount of algal growth in the tanks and remove organic waste. Not more than 50% of the tank volume was removed during siphoning.

Test compound

Flutamide (4′-nitro-3′-trifluoromethylisobutyranilide; Chemical Abstract Service no. 13311-84-7) was obtained from Mediolast (Milan, Italy). Purity was ≥99% as determined by infrared spectroscopy and high-performance liquid chromatography (HPLC). Compound stability tests done by USACEHR analytical chemists showed that flutamide was stable for one week in laboratory well water maintained at 25°C. For the duration of the 30-week test, 18 L of a 10-mg/L flutamide stock solution was prepared daily. This was accomplished by weighing out 0.18 g of flutamide and placing it in a 20-L glass container covered with aluminum foil that contained 18 L of either laboratory well water (for the first six weeks of the study) or Milli-Q–treated water (from week 7 until the end of the study at week 30). The stock solution was stirred overnight and then used to replace the current stock the following day. Leftover stock from the previous day was discarded. The flutamide stock was pumped via a peristaltic pump from the stock bottle to the solenoid dilutor system at a rate of approximately 50 ml every 5 min. The dilutor system then distributed the desired concentrations of test compound in a flow-through system to the aquaria on a 5-min cycle. Effluent was carbon filtered, and the filtered waste was analytically measured daily for chemical breakthrough.

For the first seven weeks of the test, 20-ml samples were taken daily from each of the 10 test tanks as well as from the flutamide stock bottle for flutamide analysis. Starting with week 4, daily samples were also taken from the delivery tube leading from the stock bottle. From weeks 8 through 17, test tank samples were taken biweekly. From week 18 through 30, tank samples were collected on a weekly basis; stock bottle and delivery tube samples were still collected daily. Samples for spike recovery were also taken weekly. Samples of flutamide were analyzed in triplicate using an Agilent 1100 series HPLC equipped with a diode array detector, temperature controlled autosampler, and Chemstation software (Agilent, Avondale, PA, USA). Briefly, samples were analyzed as soon as possible after receipt in the laboratory and placed in an autosampler maintained at 4°C. The column was a Hewlett Packard octadecyl dilane, 10 × 0.21 cm, 5-μm particle size (Agilent), and was maintained at 35°C using a column

heater. The mobile phase was 35% acetonitrile:65% 0.2% phosphoric acid in water, the flow rate was 0.5 ml/min, the injection volume was 100 µl, and the wavelength used for the calibration and analysis of the sample was 306 nm. The detection limit for flutamide with this method was 5 nl/L. Details of the chemical analysis procedures are contained in a technical report ([18]; http://stinet.dtic.mil/cgi-bin/GetTRDoc?AD=ADA443504&Location=U2&doc=GetTRDoc.pdf).

Laboratory exposures

Target flutamide concentrations were 0, 10, 100, 250, and 500 µg/L in an 11- or 30-week exposure time. The flutamide concentrations were selected on the basis of two previous range-finding experiments at USACEHR (authors' unpublished data). Two replicate tanks were used per concentration with 40 frogs per tank, with the intent that data from the current pilot study would be used in power analyses to determine optimal sample size for future exposure study designs. These 10 test tanks were assigned random positions in the water bath and kept in these positions throughout the study. Because it is not possible to determine the sex of the live frogs until near puberty, it was assumed that there was a 50:50 sex ratio in the early tadpoles. The frogs were exposed to the toxicant at or before NF stage 46, which is considered to be the start of sexual differentiation. Mortality and grossly apparent morbidity were checked and logged daily. Toxicity was based on qualitative evaluation of amphibian behavior and the amount of time it took to reach developmental milestones. An interim sacrifice of 74 froglets took place at 11 weeks posthatch. The froglets taken for the interim sacrifice were randomly selected from each tank; the number of frogs taken from each tank was determined by the number needed to leave a density of 30 frogs per tank for the final 30-week sacrifice. The froglets for the interim sacrifice were euthanized in laboratory well water containing 0.2% benzocaine (Sigma Chemical, St. Louis, MO, USA), and the frogs for the final sacrifice were euthanized in laboratory well water containing 0.2% tricaine methanesulfonate (MS-222) (Spectrum, New Brunswick, NJ, USA), which has been shown to be optimal for frog anesthesia [19]. Gonads were collected from the froglets during the interim sacrifice and were handled in the same manner as described here for the adults.

Tissue collection and examination

Tissues collection during the final sacrifice took place over a 4-d period on week 30 of the study. Frogs were anesthetized one at a time in a beaker of 0.2% MS-222 (tricaine methanesulfonate; Spectrum) in laboratory well water. Body weights and lengths (from snout to end of feet) were recorded, and blood was collected from each frog. The brain, liver, thyroid complex, and gonadal tissues were removed. A representative section of the liver was removed and fixed for pathology or frozen in liquid nitrogen for subsequent biochemical evaluation. Ovaries were weighed, photographed, and fixed. Testes and indeterminate kidney/gonad complexes were not weighed but were placed in Bouin's fixative.

Two 15-µl aliquots of blood to be used for plasma vitellogenin (VTG) assays were obtained from each frog by severing the hind foot and then using a micropipette to collect the blood, which was then dispensed into two microcentrifuge tubes containing 285 µl sample dilution buffer (containing phosphate-buffered saline, pH 7.4, 1% bovine serum albumin, 10 mM ethylenediaminetetraacetic acid [EDTA], 0.1% Tween® 20, and 0.5% Block Ace; Serotec, Raleigh, NC, USA) [20] on ice. All chemicals were purchased from Sigma except Block Ace. The tubes were centrifuged at 8,000 *g* for 10 min at 4°C, and the plasma was removed and flash frozen in liquid N_2, then stored at −80°C. A third aliquot of extra blood was also collected using a 250-µl heparinized capillary tube and dispensed into a 600-µl Microtainer® tube (Becton Dickinson, Franklin Lakes, NJ, USA) containing 5 µl of plasma buffer (containing 50 mM EDTA, 50 mM ethylene glycol bis[2-aminoethyl ether]-*N,N,N′N′*-tetraacetic acid) at room temperature. The tubes were centrifuged at 10,000 to 15,000 *g* for 2 min at 4°C, and the plasma was removed and stored at −80°C. Blood volumes varied according to health and size of frogs but generally were about 10 to 20 µl, and the entire blood collection process did not exceed 2 min for each frog.

Histopathology

Photographs taken of the kidney–adrenal–gonadal complexes from the male and indeterminate frogs after Bouin's fixation were used to determine where the tissues should be cut for optimum histological sectioning. The tissues were embedded in paraffin, and two to five 10-µm step sections were taken per kidney–adrenal–gonadal complex perpendicular to the long axis of the developing gonad. For the females, the ovarian egg mass was oriented to obtain the largest section possible. One representative section was taken at this orientation. All of these reproductive tissue slides were stained with hematoxylin and eosin by American HistoLabs (Gaithersburg, MD, USA).

Histopathological examinations of Bouin's- fixed reproductive organs were limited to the use of paraffin-embedded hematoxylin and eosin–stained tissues. Blind tissue evaluation was conducted using coded specimens and included an evaluation of reproductive organ development, spermatogenesis, and oogenesis. Testicular tissue sections were examined for the presence or absence of recognizable gonadal tissue, seminiferous tubule atrophy, germ cell degeneration, abnormal germ cell development, multinucleate cell formation, unusually large or small seminiferous tubule lumen, spermatocyst content, presence and/or number of testicular oocytes, sloughed germ cell located within the seminiferous tubule lumen, cellular infiltration into the interstitial space, Leydig cell hyperplasia and/or hypertrophy, and increased interstitial fibrous connective tissue. The sections were also examined for other events typically observed during spermiogenesis (nuclear condensation and elongation) and spermiation (eccentric orientation of the nucleus within the round spermatid, movement of the elongating spermatids to the periphery of the follicle cell, repositioning of the elongated spermatids toward the follicle cell nucleus, and release of the follicle cell and elongated spermatid into the tubular lumen).

Ovarian tissue sections were examined for the presence or absence of recognizable gonadal tissue, oocyte degeneration, abnormal germ cell development, presence of developing male germinal cells, differentiation of the animal and vegetal hemispheres, appearance of cortical granules, lipid accumulation in the cytoplasm, and the presence/absence, appearance, and location of nucleoli during oocyte differentiation.

A crude estimation of the volume/density measurements of ovarian components was determined by point counting. These components were previtellogenic oocytes, vitellogenic oocytes (including postvitellogenic oocytes), intercellular space, and blood vessels. Previtellogenic oocytes are defined as oocytes

that contain a relatively small nucleus and no yolk accumulation within the ooplasm. Vitellogenic oocytes are defined as oocytes characterized by the appearance of yolk granules within the ooplasm. Space was defined as the area between germ cells that did not contain any ovarian or vascular tissue. One ovarian tissue cross section per animal from each of the control and treated animals was utilized in this analysis. Tissues were examined under a binocular Axophote Zeiss microscope equipped with a bright-field condenser with a ×10 objective and a ×10 eyepiece fitted with a square lattice containing 100 intersections. The number of intersections or hits on pertinent structures over the entire tissue section was counted by a predetermined and systematic movement of sections across the grid without overlap. The volume density (Vv: the volume of the given ovarian component per unit volume of ovarian tissue) was obtained by dividing the sum of points falling on each structure (Pi) by the total number of points over the tissue (Pt). Absolute volume of each of the ovarian components (V) was determined by multiplying its volume density (Vv) by the fresh ovarian weight (Vo): Vv × Vo.

The fresh ovarian weight was utilized instead of the ovarian volume because ovarian volume measurements were not obtained prior to fixation and embedding. It is not unprecedented to utilize fixed reproductive organ weight in place of organ volume. Sinha Hikim et al. [21] have demonstrated that the volume of a fixed testis is acceptable to use for the calculation of absolute volumes of various testicular components because there is no change in testicular volume, testicular weight, or testicular specific gravity when fixed and unfixed testicular tissues are compared. As a consequence, although the calculated ovarian volumes are crude volume measurements, they do represent real changes in ovarian structure. In future studies, if quantitative histological measurements are to be obtained, ovarian volume should be determined from fresh ovarian tissues.

Paraffin-embedded livers were oriented to obtain the largest section possible for histological examination. One representative transverse section through each thyroid gland complex was obtained for histological examination. The location of these sections was determined from the postfixation photographs. Three representative sections were taken from the brains. The goal was to acquire the telencephalon at the level of the frontal organ or pineal gland, the diencephalons at the level of the optic chiasm, and the mesencephalon at the level of the hypothalamus/pituitary. Each section (3–5 μm thick) was obtained by sectioning in a rostral to caudal direction. Liver, thyroid-complex, and brain sections were all stained with hematoxylin and eosin.

Sexing of test frogs

Normal male frogs were defined as having one or both testes present at gross necropsy. The presence of testicular tissue was confirmed by histopathology. Frogs lacking testes at the time of gross necropsy were classified as "indeterminate" or having "nonrecognizable gonadal tissue" (NRGT). The absence of gonadal tissue was confirmed by histopathology.

Female frogs were defined as normal if one or both ovaries were present at the time of gross necropsy and the ovaries could be dissected out, weighed, and fixed for subsequent histopathology. Abnormal females were defined as having no weighable or removable ovarian tissue at the time of necropsy.

Vitellogenin enzyme-linked immunosorbent assays

A *Xenopus* VTG enzyme-linked immunosorbent assay (ELISA) kit (Japan EnviroChemicals, Osaka City, Osaka, Japan) was used to evaluate the plasma VTG levels in the blood of all of the study frogs. The range of detection of the kit for plasma VTG was between 0.2 and 50 ng/ml. Plasma from in-house *X. tropicalis* culture frogs was assayed for VTG levels (both male and female) prior to assaying the actual test frogs in order to determine what dilution levels, if any, would be needed for the flutamide plasma samples. From these range-finding observations, it was determined that the male plasma samples were close to the lower detection limits of the kit and should be assayed without being diluted. Female plasma samples needed to be diluted with the sample dilution buffer that was provided with the kit since their VTG levels were generally several orders of magnitude greater than the upper range of the kit. Twenty-three plasma samples plus a spike of a male plasma (for calculation of percent recovery), along with an eight-point standard curve, were run for each assay in a 96-well microtiter plate. Spikes were done by adding 40 μl of the 20-ng/ml VTG stock solution to 120 μl of male frog plasma. Percent recovery of the VTG spike was then calculated. The plasma samples for each run were selected randomly. Random samples were repeated throughout the course of the assays in order to test for reproducibility of the kits as well as reproducibility between the duplicate plasma samples from the same frog. The kit protocol was followed, and the samples were read at 450 nm on a FLUOstar OPTIMA v1.30-0 plate reader (BMG Technologies, Durham, NC, USA).

Statistical methods

The relationship between overall mean flutamide levels and the proportion of frogs with NRGT as compared to the total number of nonfemale frogs (i.e., males plus NRGT) was tested using logistic regression for an outcome variable with two categories: standard normal regression for a continuous outcome variable and the Kolmogorov–Smirnov goodness-of-fit statistic to check on the validity of the normal distribution assumption for the continuous variable regression. The relationship of other study end points and mean flutamide levels was tested using linear regression. All the tests were written in the S-plus programming language [22].

RESULTS

Test overview

Mortality in the study animals during the 30-week test (10.5%) was within the range of other flow-through exposure studies [23], and 100% of the frogs passed through metamorphosis during the predicted time period. Necropsy of the frogs, organ harvest, and blood collection for plasma VTG analysis at the end of the study were accomplished successfully with few problems. Although preliminary chemistry studies indicated that flutamide would be a good model compound for the test design and was stable in the dilution water, the target flutamide concentrations in the test tanks became difficult to maintain once the test was under way.

The data obtained from the interim sacrifice performed on days 77 and 78 of the study indicated no discernible treatment-related differences in the froglets (i.e., lengths, weights, and gonad histopathology). These data, therefore, are not analyzed or presented in this report.

On day 54 of the study, tanks 8 and 9 overflowed, releasing

Table 1. Mean and standard deviation of weights (g) and lengths (mm) of frogs

Tank no.	Mean flutamide level (μg/L)	Normal males		NRGT[a]		Normal females		Abnormal females	
		Weights (g)	Lengths (mm)	Weights (g)	Lengths (mm)	Weights (g)	Lengths (mm)	Weights (g)	Lengths (mm)
2	0 (2)	9.1 (0.9)	90 (3)	16.6[b]	102[b]	15.2 (3.0)	102 (6)	9[b]	94[b]
1[c]	1 (4)	9.5 (1.5)	90 (4)	11.6 (1.7)	92 (2)	16.1 (3.1)	103 (6)	9.7 (1.2)	90 (2)
3	8 (6)	9.0 (1.5)	89 (4)	9.5 (3.7)	90 (7)	13.8 (3.0)	100 (5)	11.1 (1.9)	92 (5)
4	9 (7)	8.9 (1.2)	89 (4)	11.2 (2.4)	95 (1)	15.2 (3.4)	100 (6)	12.9 (1.5)	105 (9)
6	52 (20)	9.5 (1.8)	91 (4)	12.2 (0.9)	99 (1)	12.9 (1.6)	97 (3)	9.5[b]	92[b]
5	53 (20)	9.1 (1.6)	89 (5)	10.5 (2.0)	92 (2)	13.3 (1.8)	100 (7)	4.6[b]	72[b]
8	130 (68)	10.4 (2.4)	90 (6)	12.8 (1.8)	97 (2)	13.9 (2.9)	97 (5)	11.9[b]	93[b]
7	152 (65)	10.4 (1.2)	91 (3)	8.6 (2.1)	90 (6)	13.9 (2.3)	98 (5)	9.3 (2.6)	90 (3)
9[d]	199 (150)	10.4 (1.6)	92 (2)	11.0 (1.5)	93 (6)	13.3 (2.5)	97 (3)	11.1 (3.1)	94 (5)
10	220 (143)	8.9 (2.7)	86 (7)	10.3 (3.1)	92 (7)	12.8 (2.9)	95 (4)	12.1 (2.7)	97 (5)

[a] Nonrecognizable gonadal tissue.
[b] Standard deviation not calculated because $n = 1$.
[c] Control tank 1 accidentally exposed to 22 μg/L flutamide for several hours after three weeks into study.
[d] Tank 9 had a combination of frogs from tanks 8 and 9 from week 7 until week 30 as a result of an overflow.

some of the frogs into the water bath. Reasons for the overflow were clogging of the drain in one tank and the unintentional elevation of the drain pipe during cleaning in the other tank. Because the source tank for the individual frogs was not known when they were captured, the frogs were simply combined and placed back into tank 9. The labeling of tank 9 became "250 + 500," indicating that it contained a combination of frogs exposed to nominal concentrations of 250 and/or 500 μg/L.

Analytical chemistry results

The target concentration for the flutamide stock solution was 10 mg/L. Measured concentrations were variable in the tanks and ranged from 10 to 56% lower than the nominal levels set for each tank. Although stability studies of flutamide in USACEHR laboratory well water prior to the start of the study indicated that flutamide was stable, the actual concentrations of the flutamide stock solution began to drop precipitously beginning at day 40 of the study, causing average tank concentrations to fall as well. Stock solution concentrations stabilized after deionized water was used to replace well water as a diluent on day 45, but test tank concentrations continued to fall. Although the exact cause of the drop in mean flutamide concentrations is not known, a likely explanation is microbial degradation. Because of the low water solubility of flutamide, it was not practical to compensate for flutamide degradation in the test tanks by increasing the turnover rate of the diluter. Additionally, a leak in the flutamide stock solution tube on day 26 resulted in control tank 1 receiving flutamide for approximately 4 h before the leak was discovered and repaired. The maximum measured mean flutamide concentration for that day was 22 μg/L. Because of this accidental exposure, tank 1 data were kept separate from control tank 2 data during statistical analysis.

Summary of body weight and lengths

The mean body weights and lengths from the study are summarized in Table 1. Among the normal male frogs, a slight increase in mean body weight with increasing mean flutamide concentration was observed ($p = 0.008$), but no trend was detected with mean body length and mean flutamide level ($p = 0.34$). Among the NRGT frogs, no significant relationships between mean body length or weight and mean flutamide concentration were observed.

Among the normal female frogs, both the mean body length and weight significantly decreased with increasing mean flutamide concentration ($p = 0.001$ and 0.009, respectively). However, among the abnormal female frogs, no significant relationships between mean body length or weight and mean flutamide concentration were observed.

Gross testis morphology

Concentration-related effects of the 30-week flutamide exposure were observed for the NRGT sexual morphological category. Table 2 summarizes these effects based on observations made at the time of necropsy as well as subsequent histopathological examination of the gonadal tissue slides. Considering the antiandrogenic effects of flutamide, it was assumed the NRGT frogs were genetic males. Using logistic regression analysis, there was a statistically significant increase ($p = 0.004$) in the proportion of NRGT frogs to the total number of male and NRGT frogs with increasing mean flutamide concentrations. Two of the frogs in tank 2 and one frog in tank 4 had only one testis but were classified as normal males.

Control tank 1, which accidentally received flutamide briefly, contained 28.6% NRGT animals (four frogs). In contrast, control tank 2 had only 5.5% NRGT animals (one frog). This observation suggests that a transient flutamide exposure during critical developmental time points might disrupt the development of male gonads. Since only 14 male frogs were involved, the data set is too small for reliable statistical analysis, and the issue of a critical time period for flutamide exposure cannot be statistically evaluated from the existing study design.

Qualitative microscopic analysis of testes

The seminiferous tubules and interstitial compartments of the testes were examined for flutamide-related effects. The arrows in Figure 1 show the seminiferous tubules in a control animal; they are highly convoluted and lined with spermatocysts containing germ cells undergoing spermatogenesis. Each spermatocyst contains a cluster of germ cells at the same spermatogenic phase of development. Differentiated spermatocysts, which line the seminiferous tubule lumen, contain clones of germ cells at different spermatogenic phases of development. All phases of spermatogenesis (spermatocytogenesis, meiosis, and spermiogenesis) appeared normal in control and flutamide-treated frogs. Early round spermatids containing condensed nuclei were seen throughout the spermatocyst; sper-

Table 2. Distribution of sexes of frogs in treatment tanks

Tank no.	Mean flutamide level (μg/L)	Total frogs	Normal males[a]	NRGT[b]	% NRGT[c]	Normal females[d]	Abnormal females[e]	% Abnormal females
2	0	29	17[f]	1	5.5	10	1	9.1
1[g]	1	30	10	4	28.6	13	3	18.7
3	8	26	14	2	12.5	8[h]	2	20
4	9	29	13[i]	4	23.5	10	2	16.7
6	52	30	15	3	16.7	11	1	8.3
5	53	29	17[i]	3	15	8	1	11.1
8	130	18	5	5	50	7	1	12.5
7	152	30	11	3	21.4	12	4	25
9[j]	199	30	11	5	31.2	10[k]	4	28.6
10	220	29	5	5	50	14[h]	5	26.3

[a] Normal males defined as having both testes unless otherwise indicated.
[b] Nonrecognizable gonadal tissue.
[c] The denominator was derived from the sum of normal males and NRGT.
[d] Normal females defined as having both ovaries unless otherwise indicated.
[e] Abnormal females defined as having no weighable ovaries.
[f] Two frogs in this tank had only one testis.
[g] Control tank 1 accidentally exposed to 22 μg/L flutamide for several hours after three weeks into study.
[h] One frog in these tanks had only one ovary.
[i] One frog in these tanks had only one testis.
[j] Tank 9 had a combination of frogs from tanks 8 and 9 from week 7 until week 30 as a result of an overflow.
[k] Two frogs in this tank had only one ovary.

matids were positioned around the periphery, and in later stages, open spermatocysts and elongated spermatids were positioned within crypts formed by the follicle cell. The nuclei of these elongated spermatids were seen positioned adjacent to the follicle cell nucleus (Fig. 1).

Germ cell degeneration was not observed during the meiotic phase of spermatogenesis in either the control group or any of the flutamide treatment groups. Testicular oocytes, sloughed germ cells lying within the seminiferous tubule lumen, cellular infiltration of the interstitial space, and an increase in interstitial fibrous connective tissue were occasionally observed in treated and untreated frogs, but the effects were not concentration related. Although the frogs were examined for adverse effects such as seminiferous tubule atrophy, germ cell degeneration, abnormal germ cell development, multinucleate cell formation, seminiferous tubules with unusually large or small lumen, and Leydig cell hyperplasia or hypertrophy, none of these effects were observed. In general, the interstitial cell compartments of both the control and the flutamide-treated animals were indistinguishable. Leydig cells and occasional collecting ducts were observed in the interstitial space of both the control and the flutamide-treated animals. Histological examination of the gonadal region of frogs classified as NRGT revealed only the kidneys and the aorta or other large blood vessels and thin sheets of fibrous connective tissue located along the midline between each kidney. No evidence of gonadal tissue was observed in these animals.

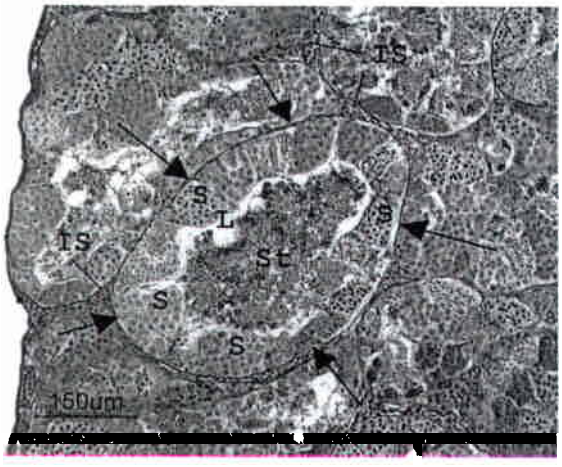

Fig. 1. Photomicrograph of testes in control frog. IS = interstitial space, arrows = boundary of seminiferous tubule, S = spermatocyst, L = lumen, St = spermatids released into lumen of seminiferous tubule.

Gross morphology of ovaries

The percentage of abnormal female frogs (defined as females without removable and weighable ovarian tissue) seemed to increase in each treatment group with increasing mean flutamide concentrations after 30 weeks, but this increase was not statistically significant ($p = 0.15$).

The mean ovary weight among normal female frogs significantly decreased with increasing mean flutamide concentrations ($p = 0.001$); this effect remained significant after normalizing the ovary weights for body weights ($p = 0.001$). However, ovary weight variability within the control and flutamide-exposed tanks was quite high (coefficient of variation = 41–84%).

Quantitative microscopic analysis of ovaries

The ovaries of the control and flutamide treated frogs contained oocytes at all stages of development as defined by Dumont [24]. Blood vessels, connective tissue, and variable amounts of space could be found interspersed between the developing oocytes (Fig. 2A). An examination of ovarian tissues from a number of flutamide-treated animals suggested that these tissues contained more previtellogenic/very early vitellogenic follicles than the control group (Fig. 2B). As a consequence of this observation, a quantitative morphometric assessment of the tissue sections was conducted.

Vitellogenic oocytes occupied approximately 65 and 64% of the ovarian volume density from control animals in tanks 1 and 2, respectively. No trend was observed with increasing mean flutamide concentrations. Previtellogenic follicles oc-

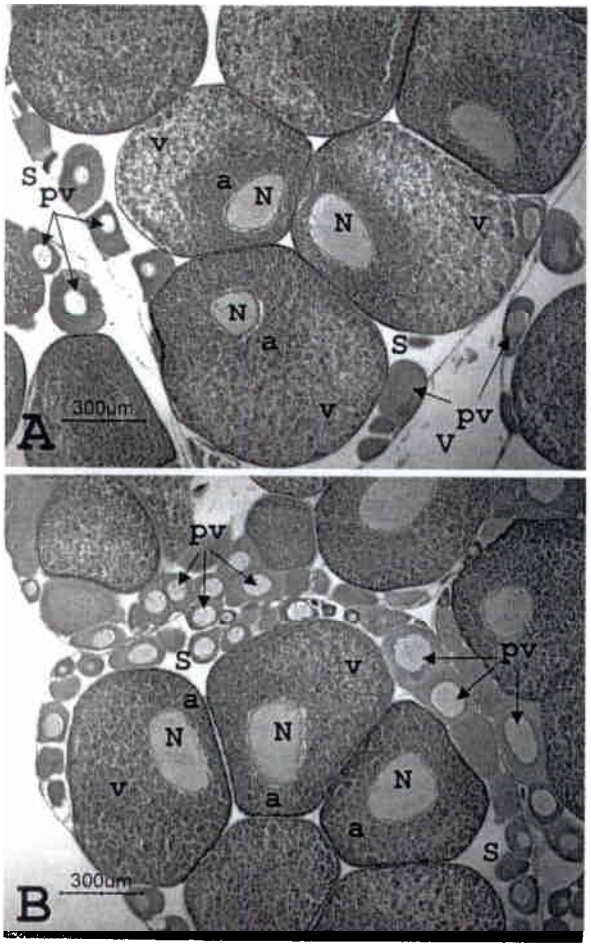

Fig. 2. **(A)** Photomicrograph of oocytes in control frog. S = space, pv = previtellogenic oocyte, V = blood vessel, n = nucleus, a = animal pole, v = vegetal pole. **(B)** Photomicrograph of oocytes in flutamide-exposed frog.

cupied approximately 9% of the ovarian volume density from control animals in both tanks 1 and 2, and the results from the flutamide-exposed tanks were not significantly different, ranging from 9 to 18% of the ovarian volume density. The volume density of the blood vessels and intercellular space (the area between germ cells that did not contain any ovarian or vascular tissue) was variable.

The absolute volume of the vitellogenic oocytes decreased as mean flutamide concentration increased, but this observation is consistent with the decreasing body and ovarian weights of the flutamide-exposed female frogs. The absolute volume of the previtellogenic follicles did not vary from the control values as the mean flutamide levels increased. No concentration-related response in the absolute volume of the blood vessels was observed over the dose levels utilized in the study. The absolute volume of the intercellular space decreased with the decreasing ovarian weight and increasing mean flutamide concentrations, which is expected with smaller ovarian masses.

Necropsy observations of nongonadal tissue

Among the normal male frogs, the mean liver weights normalized for body weight increased significantly with increasing concentrations of flutamide (p = 0.042). However, no significant relationship between normalized liver weight and mean flutamide concentration was observed for the NRGT frogs. Among the normal female frogs, no differences were seen in normalized liver weight with increasing mean flutamide concentrations (p = 0.84).

Table 3. Mean and standard deviation of plasma vitellogenin levels (µg/ml)

Mean flutamide level (µg/L)	Normal males[a]	NRGT[b]	Normal females[c]	Abnormal females[d]
0	0.062 (0.136)	1.001[e]	222 (94.3)	0.64
1	0.025 (0.073)	0.379 (0.288)	312 (233)	26.3 (44.1)
8	0.010 (0.012)	0.039 (0.019)	171 (69.1)	6.13 (7.40)
9	0.012 (0.013)	0.020 (0.015)	250 (78.4)	0.214 (0.056)
52	0.042 (0.073)	0.031 (0.026)	227 (107)	0.893[e]
53	0.100 (0.346)	0.233 (0.380)	216 (75.8)	6.01[e]
130	0.059 (0.084)	0.074 (0.093)	276 (47.9)	28.5[e]
152	0.219 (0.646)	0.004 (0.007)	254 (53.6)	40.8 (75.9)
199	0.027 (0.025)	0.043 (0.055)	229 (129)	97.3 (159)
220	0.015 (0.009)	5.17 (9.89)	209 (115)	49.2 (99.4)

[a] Normal males defined as having both testes unless otherwise indicated.
[b] Nonrecognizable gonadal tissue.
[c] Normal females defined as having both ovaries unless otherwise indicated.
[d] Abnormal females defined as having no weighable ovaries.
[e] Standard deviation not calculated because n = 1.

Histopathology of nongonadal tissue

No histopathological abnormalities were observed in the livers and brains of the frogs from the flutamide-exposed tanks. Although the sectioning protocol used for the brains and livers provided representative sections of these organs for examination, the sectioning protocol used for the thyroid complex was unsuccessful. No thyroid tissue was found in the expected sections. Further methods development needs to be done on the sectioning of *X. tropicalis* thyroids.

ELISA VTG results

The VTG ELISA kit that was used to measure the plasma VTG levels in the *X. tropicalis* for this study was designed and marketed for *X. laevis*. Methods were developed to adapt the kit for use in *X. tropicalis*, but the kit was not validated for *X. tropicalis* prior to the final testing. Table 3 is a summary of the average plasma VTG levels for each treatment tank of frogs. The VTG levels were four to five orders of magnitude greater in the normal females compared to the normal males and NRGT frogs. For the most part, abnormal females had VTG levels that were one to two orders of magnitude lower than normal females.

Slight but statistically significant increases in VTG levels were found as mean flutamide levels increased in the combined group of normal males and NRGT frogs (p = 0.035). In the normal female frogs, the VTG levels decreased slightly with statistical significance ($p < 0.001$) along with increasing flutamide exposure levels, which may be related to decreased ovary and liver weights seen in normal females.

DISCUSSION

In the present study, we have demonstrated statistically significant dose–response effects in *X. tropicalis* using a 30-week tier 2 assay for endocrine disruption. We emphasize that the purpose of this pilot study was not to assess the toxicity

of the antiandrogen flutamide but rather to assess the design and efficacy of a 30-week exposure protocol using *X. tropicalis*. Difficulties encountered with implementing the exposure protocol have provided valuable insights and helped to identify problems that must be solved before a tier 2 amphibian assay can be further developed and validated.

The greatest difficulty encountered while executing the exposure protocol was the maintenance of nominal concentrations of flutamide in the test tanks. Although we had determined that flutamide was stable in laboratory well water, flutamide stock concentrations began to drop precipitously beginning on day 40 of the study, thereby causing the test tank concentrations to fall as well. Although the exact cause of the drop in mean flutamide concentrations was not known, the most likely explanation was that the diluter and test tank systems were colonized by bacteria that degraded the flutamide. It was not practical to sterilize the diluters, and we have found that more frequent cleaning of the test tanks would have increased mortality and affected frog health. Because of the low solubility of flutamide in water, it was not practical to compensate for flutamide degradation by increasing the turnover rate of the diluter. A carrier solvent with the flutamide was not used because the addition of an appropriate solvent control was not feasible after the exposure was ongoing for 40 d. Detailed information on tank concentration over time is available in a technical report [18].

The flutamide concentration issue is chemical specific, whereas the overflow of the tanks during the exposures is directly relevant to the exposure protocol. Consequently, we are taking steps to reduce the possibility of tank overflows. Along with routine checks of the tank water levels and the unclogging of drains, we have made clear Plexiglas lids, with holes for feeding and aeration, for the tops of the tanks. Since frog density directly contributes to the amount of debris (mostly waste food and fecal matter) in the tanks and the clogging of drains, we are studying the optimum frog density with respect to minimizing debris in tanks.

The difficulty of maintaining the mean flutamide concentrations underscores an important issue regarding long-term aquatic exposures to detect endocrine toxicity: the measurement of nominal, free, internal, and target exposure concentrations. Escher and Hermens [25] discuss the distinction between these exposures and advocate using internal or target exposure concentrations to improve research in environmental (aquatic) toxicology and to assess risks. The internal and target exposure concentrations are particularly important with aquatic organisms because the bioavailability of the EDC may not be known and the route of exposure may affect the metabolism of an EDC. With amphibians like *X. tropicalis*, the uptake of flutamide is most likely through dermal exposure, which means that it may not be immediately metabolized by the liver and has important implications in toxicokinetic processes. Furthermore, flutamide is metabolically activated to 2-hydroxyflutamide in the liver by CYP1A2 [26], and the expression of a CYP1A2 homologue in *X. tropicalis* has not been reported. Although desirable, the measurement of internal and target exposure concentrations of an EDC in *X. tropicalis*, along with CYP enzyme expression, would present additional challenges in study design and analytical chemistry.

The observed flutamide effects in our pilot study were highly variable and involved mostly changes in well-defined endpoints such as body lengths and weights, organ weights, and gross pathology. Statistically significant changes were not observed between the control and exposed groups in the histopathology of tissues. It should be noted, however, that a baseline for the normal histology of *X. tropicalis* tissue, a necessary reference for evaluating histopathology, has not been fully established. For the thyroid, the development of a technique is needed to properly section them. We had originally planned to conduct sperm counts, but because the final sacrifice was very time consuming and resource intensive, we decided to forego the procedure for this pilot study. We also planned to weigh the testes, but during final sacrifice, we left them attached to the kidney/adrenal complex. This made removal of the testes less time consuming, prevented tissue damage during excision, and reduced the risk of losing or damaging the smaller testes.

On the level of gross pathology, statistically significant differences in the percentage of NRGT frogs were observed with increasing exposure across the groups. We presumed that the NRGT frogs were genetically programmed to become males and that flutamide exposure inhibited androgen uptake and/or nuclear binding of androgen in the target tissues, resulting in the disruption of gonadal development in some male frogs. However, epigenetic factors resulting from temperature, pH, ionic composition at time of breeding, and diet have been shown to influence amphibian sex determination [27,28]. Unfortunately, a genetic marker for sex determination in *X. tropicalis* has not yet been identified, which makes confirmation of such effects from an EDC exposure problematic.

From work done in our laboratory during the collection of baseline histology data, we have rarely encountered NRGT animals (authors' unpublished data), which raises the question as to why control tank 1 had 5.5% NRGT animals. Ashby [29] asserts that low-dose endocrine toxicities are particularly difficult to detect because of the inherent variability within control groups of the parameters being studied. This variability within control animals may be influenced by a variety of factors, such as stress from overcrowding and naturally occurring endocrine-active compounds in food. Ashby recommends establishing historical control databases for the parameters under study and linking them to experimental influences that affect control variability. To that end, we are currently examining the influences of frog density and water turnover rates in test tanks with respect to several parameters, including the weights, gross anatomy, and histology of gonadal tissues along with other parameters. We are also reviewing the study design for statistical power considerations with particular emphasis on the number of control and test tanks and the frog density within the tanks.

The increased percentage of NRGT frogs in one control tank receiving the serendipitous exposure to flutamide suggests that a one-time exposure during sensitive life stages may be sufficient to alter sexual development in adults, but our pilot study was not designed to evaluate such effects. Although the earliest life stages may be more sensitive to an EDC, many traditional endpoints of toxicity are not likely to be detected until sexual maturity is reached. The indiscernible differences between groups from the interim sacrifice in our pilot study demonstrate this dilemma. As suggested by Daston et al. [30], however, these traditional endpoints are likely to be preceded by changes in gene expression that may be more immediate and persistent. The predictive value of gene expression changes for such phenotypic changes in animals is undetermined and will require further research. Since the *X. tropicalis* genome has been sequenced and microarrays have been con-

structed for *Xenopus* species [31], we believe it will be feasible to include gene expression changes among the effects for evaluation in future studies.

Others have shown that flutamide exposure increases plasma VTG levels in male fathead minnows [32] and that flutamide treatment blocks androgen-induced VTG decrease in rainbow trout [33]. For this reason we chose plasma VTG as a biomolecular endpoint. The assay kit used here to detect VTG was designed for use in *X. laevis* but for our purposes appeared to be adaptable for use in *X. tropicalis*. Normally at low or undetectable levels in males, plasma VTG is an egg-yolk precursor protein that is induced by estrogen and produced by the liver [34]. Environmental estrogenic compounds can stimulate the liver to produce VTG in males and subsequently elevate the plasma levels of this protein [35]. Therefore, VTG is more suitable as a biomarker for estrogenic compounds rather than antiandrogenic compounds, such as flutamide, making the VTG plasma assay results of this study not unexpected. The slightly increasing plasma VTG levels in the combined group of male and NRGT frogs with increasing mean flutamide concentrations could be due to the slight increase in the liver weights of these animals. Conversely, slight decreases in plasma VTG levels in the female frogs with increasing mean flutamide concentrations could be explained by decreases in ovary and liver weights of these animals. For our purposes, the assay proved to be reproducible and sensitive and resulted in the design of a VTG biomarker protocol for further validation and use in future studies with *X. tropicalis*.

To estimate the power for a similar future study to find a statistically significant effect of flutamide, we will use the results from this study. For example, if we consider the percentage of males classified as NGRT, the estimated logistic regression slope was 0.006 with a standard error of 0.0024. Because the standard error is inversely proportional to the square root of the number of replicates ($n = 2$ in our study), the standard error using three replicates would be $0.0024 \cdot \sqrt{2/3}$, and the standard error using five replicates would be $0.0024 \cdot \sqrt{2/5}$. Assuming that the estimated slope follows a Gaussian distribution with mean = 0.006, the estimated power of an experiment with five replicates is 0.757, with 10 replicates is 0.837, and with 17 replicates is 0.900, indicating that it may be possible to overcome the problem of high variation seen in this study by increasing the number of replicates.

In summary, we recommend that an extensive baseline study be conducted to establish values for unexposed *X. tropicalis* raised under the same conditions as exposed frogs. Additionally, we plan to consult with a statistician on study design to ensure that we have sufficient statistical power to overcome the high variability seen in this study. We also plan to develop gene expression endpoints and are considering the use of microarrays to examine gene expression changes en masse. Finally, we are investigating measuring tissue levels of the test chemical, but constraints exist in the amount of tissue that is obtainable and the analytical limits of detection.

Despite some unforeseen difficulties over the 30-week exposure period to flutamide, we were able to determine an endocrine-disrupting effect from flutamide exposure. The current pilot study represents the longest reported laboratory investigation of an EDC using the model amphibian *X. tropicalis*. However, at this phase of development, the test protocol is not ready for rigorous interlaboratory validation. We are continuing the next phase of the 30-week tier 2 assay for the effects of endocrine-disrupting chemicals on *X. tropicalis* through incorporation of our recommendations and lessons learned.

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REFERENCES

1. Diffley JF. 2004. Regulation of early events in chromosome replication. *Curr Biol* 14:R778–R786.
2. Blow JJ. 2001. Control of chromosomal DNA replication in the early Xenopus embryo. *EMBO J* 20:3293–3297.
3. Dale L, Jones CM. 1999. BMP signalling in early Xenopus development. *Bioessays* 21:751–760.
4. Brandli AW. 1999. Towards a molecular anatomy of the Xenopus pronephric kidney. *Int J Dev Biol* 43:381–395.
5. Bevan CL, Porter DM, Prasad A, Howard MJ, Henderson LP. 2003. Environmental estrogens alter early development in *Xenopus laevis*. *Environ Health Perspect* 111:488–496.
6. Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc Natl Acad Sci USA* 99:5476–5480.
7. Lee SK, Veeramachaneni DN. 2005. Subchronic exposure to low concentrations of di-*n*-butyl phthalate disrupts spermatogenesis in *Xenopus laevis* frogs. *Toxicol Sci* 84:394–407.
8. Tavera-Mendoza L, Ruby S, Brousseau P, Fournier M, Cyr D, Marcogliese D. 2002. Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. *Environ Toxicol Chem* 21:527–531.
9. Hayes T, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A. 2003. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): Laboratory and field evidence. *Environ Health Perspect* 111:568–575.
10. Mayer LP, Dyer CA, Propper CR. 2003. Exposure to 4-*tert*-octylphenol accelerates sexual differentiation and disrupts expression of steroidogenic factor 1 in developing bullfrogs. *Environ Health Perspect* 111:557–561.
11. Amaya E, Offield MF, Grainger RM. 1998. Frog genetics: *Xenopus tropicalis* jumps into the future. *Trends Genet* 14:253–255.
12. Carruthers S, Stemple DL. 2006. Genetic and genomic prospects for *Xenopus tropicalis* research. *Semin Cell Dev Biol* 17:146–153.
13. Song MO, Fort DJ, McLaughlin DL, Rogers RL, Thomas JH, Buzzard BO, Noll AM, Myers NK. 2003. Evaluation of *Xenopus tropicalis* as an alternative test organism for frog embryo teratogenesis assay—Xenopus (FETAX). *Drug Chem Toxicol* 26: 177–189.
14. Hirsch N, Zimmerman LB, Grainger RM. 2002. Xenopus, the next generation: *X. tropicalis* genetics and genomics. *Dev Dyn* 225:422–433.
15. Richardson PM, Chapman J. 2003. The *Xenopus tropicalis* genome project. *Current Genomics* 4:645–652.
16. Hirsch N, Zimmerman LB, Grainger RM. 2002. Xenopus, the next generation: *X. tropicalis* genetics and genomics. *Dev Dyn* 225:422–433.
17. Hubrecht-Laboratorium EI, Nieuwkoop PD, Faber J. 1967. *Normal Table of* Xenopus laevis *(Daudin): A Systematical and Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis.* North-Holland, Amsterdam, The Netherlands.
18. Knechtges PL, Sprando RL, Brennan LM, Miller MF, Kumsher

DM, Dennis WE, Brown CC, Clegg ED. 2006. Assessment of protocol designed to detect endocrine disrupting effects of flutamide in *Xenopus tropicalis*. USACEHR-TR-0601. Defense Technical Information Center, Fort Belvoir, VA, USA.
19. American Veterinary Medical Association Panel on Euthanasia. 2001. 2000 report of the AVMA panel on euthanasia. *J Am Vet Med Assoc* 218:669–696.
20. Mitsui N, Tooi O, Kawahara A. 2003. Sandwich ELISAs for quantification of *Xenopus laevis* vitellogenin and albumin and their application to measurement of estradiol-17 beta effects on whole animals and primary-cultured hepatocytes. *Comp Biochem Physiol C Toxicol Pharmacol* 135:305–313.
21. Sinha Hikim AP, Bartke A, Russell LD. 1988. Morphometric studies on hamster testes in gonadally active and inactive states: Light microscope findings. *Biol Reprod* 39:1225–1237.
22. Everitt B, Everitt B. 1994. *Statistical Methods in Medical Investigations*. E. Arnold, London, UK.
23. Pickford DB, Hetheridge MJ, Caunter JE, Hall AT, Hutchinson TH. 2003. Assessing chronic toxicity of bisphenol A to larvae of the African clawed frog (*Xenopus laevis*) in a flow-through exposure system. *Chemosphere* 53:223–235.
24. Dumont JN. 1972. Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J Morphol* 136:153–179.
25. Escher BI, Hermens JL. 2004. Internal exposure: Linking bioavailability to effects. *Environ Sci Technol* 38:455A–462A.
26. Shet MS, McPhaul M, Fisher CW, Stallings NR, Estabrook RW. 1997. Metabolism of the antiandrogenic drug (flutamide) by human CYP1A2. *Drug Metab Dispos* 25:1298–1303.
27. Eggert C. 2004. Sex determination: The amphibian models. *Reprod Nutr Dev* 44:539–549.
28. Reeder AL, Foley GL, Nichols DK, Hansen LG, Wikoff B, Faeh S, Eisold J, Wheeler MB, Warner R, Murphy JE, Beasley VR. 1998. Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). *Environ Health Perspect* 106:261–266.
29. Ashby J. 2003. Problems associated with the recognition and confirmation of low-dose endocrine toxicities. *Nonlinearity in Biology, Toxicology, and Medicine* 1:439–453.
30. Daston GP, Cook JC, Kavlock RJ. 2003. Uncertainties for endocrine disrupters: Our view on progress. *Toxicol Sci* 74:245–252.
31. Chalmers AD, Goldstone K, Smith JC, Gilchrist M, Amaya E, Papalopulu N. 2005. A *Xenopus tropicalis* oligonucleotide microarray works across species using RNA from *Xenopus laevis*. *Mech Dev* 122:355–363.
32. Jensen KM, Kahl MD, Makynen EA, Korte JJ, Leino RL, Butterworth BC, Ankley GT. 2004. Characterization of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow. *Aquat Toxicol* 70:99–110.
33. Shilling AD, Williams DE. 2000. The non-aromatizable androgen, dihydrotestosterone, induces antiestrogenic responses in the rainbow trout. *J Steroid Biochem Mol Biol* 74:187–194.
34. Arukwe A, Goksoyr A. 2003. Eggshell and egg yolk proteins in fish: Hepatic proteins for the next generation: Oogenetic, population, and evolutionary implications of endocrine disruption. *Comparative Hepatology* 2:4.
35. LeBlanc GA, Bain LJ. 1997. Chronic toxicity of environmental contaminants: Sentinels and biomarkers. *Environ Health Perspect* 105(Suppl. 1):65–80.